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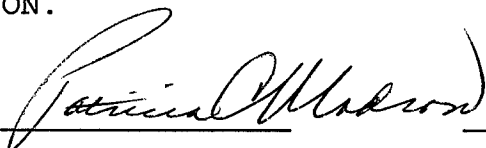
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Petr V. Hanochka 10/14/98
PI - Signature Date

Annual Report**Bone Factors Regulating the Osteotropism of Metastatic Breast Cancer**

Principal Investigator:

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Children's Hospital, Boston, MA 02115Table of Contents

<u>Section</u>	<u>Page</u>
Report Documentation Page (Form SF298)	2
Foreword (Form)	3
Table of Contents	4
Abstract (Repeated from Face Page)	5
Introduction	5
Body of Report	6
Overview of "Statement of Work"	6
Progress on Technical Objective #1	6
Methods	6
Results	8
Progress on Technical Objective #2	10
Methods	10
Results	10
Progress on Technical Objective #3	11
Methods	11
Results	11
Discussion	13
Conclusions	15
References	16
Appendix	19-21
Abstract to Orthopedic Research Society (scheduled for oral presentation)	

Abstract (Repeated from Face Page)

This project seeks to identify the critical molecular interactions and cellular processes responsible for the preferential establishment of breast cancer metastases in the human skeleton. We are testing the hypothesis that malignant breast adenocarcinoma cells subvert the cooperative paracrine interactions between normal bone cells, endothelial cells, and the extracellular matrix in order to establish metastatic foci. We have developed strong preliminary data showing mimicry of the osteoblast phenotype by invasive breast adenocarcinoma cells; this mimicry includes a pattern of gene expression which may explain the osteotropism (homing and metastatic growth in bone) of breast cancer. In model human cell lines, and sublines recovered from bone metastases in nude mice, the inappropriate expression of the "bone specific" transcription factor CBFA1 appears to correlate with the degree of malignancy, and this probably drives the osteoblast mimicry; normal breast tissue does not express CBFA1.

Introduction

Osteotropism of metastatic breast cancer (i.e., the predisposition of malignant adenocarcinoma cells to lodge in bone and establish painful osteolytic metastases) is the focus of this Project, and has direct relevance to three of the goals of the USAMRMC/DOD Breast Cancer Research Program:

1. We are defining changes in cell and molecular functions accounting for the development and progression of breast cancer.
2. The findings could be of potential use in guiding diagnosis, prevention, and treatment.
3. The research targets a major economic element (the costs of hospitalization and clinical management of skeletal morbidity) and could lead to more cost-effective health care delivery.

Malignant breast cancer cells exploit the normal bone homeostatic mechanisms in 3 ways for their own benefit: 1) secretion of PTH-rp and other factors stimulates osteolysis, creating space for metastatic tumor growth; 2) growth factor release during pathologic osteolysis drives adenocarcinoma cell proliferation; and 3) angiogenesis caused by osteolysis provides increased blood flow and a plentiful supply of nutrients to growing metastases. Partial mimicry of the osteoblast phenotype by malignant breast adenocarcinoma cells¹⁻³ is hypothesized to allow the expropriation of normal osteoblast-osteoclast signaling pathways for the exclusive benefit of growing tumor metastases in bone. Release of growth factors and rapid angiogenesis occurs in the immediate vicinity of an active osteoclast.^{4,5} Osteoblast-derived bone sialoprotein (BSP), osteopontin (OPN), and osteocalcin (OC) are required for osteoclast attachment and activation,⁵⁻¹⁰ and expression of BSP by primary human breast adenocarcinoma is a recent predictor of metastasis.³ Little is known about the regulation of such events. We are using *in vitro* and *in vivo* models to characterize this important pathological mimicry by malignant breast cancer cells.

Our ongoing and planned experiments should provide insight into the biology of painful bone metastasis in invasive breast cancer, offering hope for innovative therapy. Given the unchanging survival outlook for patients with metastatic bone disease, any significant improvement in the quality of remaining life would stand as an important therapeutic effect.

Body of Report**Overview of "Statement of Work"**

There are three Technical Objectives comprising the original Statement of Work for this project. On the following pages, results have been organized according to these Technical Objectives and the specific, numbered Tasks within each section on which progress has been made.

Progress on Technical Objective #1

Technical Objective 1: Examine the regulation of malignant human breast adenocarcinoma cell lines by bone extracellular matrix proteins and by normal osteoblasts *in vitro*.

Tasks 2 and 3: Develop baseline data on cell proliferation and gene expression by the MCF-7 and MDA-MB-231 human breast adenocarcinoma cell lines. Establish co-cultures of osteoblasts and breast adenocarcinoma cells to study cellular interaction and the process of osteoblastic mimicry. That is, define the expression of bone matrix proteins (BSP, OPN, OC) and a bone-specific transcription factor (CBFA1) by adenocarcinoma cells.

Methods: The primary analysis involves comparison of adenocarcinoma cell gene expression and proliferation in the presence and absence of normal osteoblasts. A proven method for this interactive analysis of soluble factors is trans-well co-culture, where tumor cells growing on a semi-permeable membrane are placed in proximity to the osteoblast monolayer culture. Direct cell-cell contact configurations allow the analysis of cell-matrix and cell-cell (adenocarcinoma-osteoblast) contact in the regulation of gene expression and proliferation.

Gene Expression Markers: Our prime focus is to define the regulation of adenocarcinoma gene expression by bone factors. A secondary focus is modulation of the osteoblast phenotype by the tumor cells. Standard methods include RIA, EIA, western blot, northern blot, RT-PCR, in situ hybridization, and immunohistochemistry, depending on the sensitivity and degree of cellular resolution required. RNA/RT-PCR RNA extraction was performed using RNeasy Mini Kit (Qiagen, CA). Primers were synthesized to unique regions in the osteocalcin, CBFA1, and GAPDH human cDNA sequences reported in the Genbank (Table 1). PCR reaction cycle conditions consisted of denaturation at 93°C for 60s, primer annealing at 61°C for 60s, extension at 72°C for 60s, and a final extension at 72°C for 7 min in a MJ Research (PTC-100) programmable thermal-cycler. Samples were amplified for 25 cycles. UV photography and densitometry of ethidium bromide stained gels was performed. Semi-quantification was achieved by comparing the amplicon intensities of both CBFA1 and osteocalcin to GAPDH.

Table 1: RT-PCR PRIMERS

	Forward Primer	Reverse Primer	Size (bp)
HumanOC	5'-ATGAGAGCCCTCAGACTCCTC-3'	5'-CGGGCCGTAGAAGCGCCGATA-3'	294
HumanCBFA1	5'-TTACTTACACCCCGCCAGTC-3'	5'-TTCAATATGGTCGCCAAACA-3'	375

Competitive RT-PCR: Cells are plated to confluence and then passaged using 0.5% Trypsin (Gibco), washed with medium to neutralize the trypsin, and centrifuged at 3000xg. RNA extraction was performed using the RNeasy Mini Kit (Qiagen, CA). Purified RNA was quantified using UV spectrophotometry (260/280nm). Genomic DNA contamination is eradicated by treating with RQ1-DNase. DNase treated RNA subsequently is reverse transcribed to cDNA. Construction and synthesis of internal standard RNA: The 294 bp PCR product and CBFA1 PCR products will undergo restriction enzyme digestion with NlaIII restriction enzyme. This cleavage will result in 3 fragments. The two outer fragments of each product will be purified and ligated. The ligation products will be 90 and 104 bp smaller than the original template. The ligation products will be amplified by PCR, ligated in the pCR II (InVitrogen, CA) transcription vector containing the T7 polymerase promoter and cloned into E. Coli DH5 α . After verifying the presence and orientation of the insert, the plasmid will be purified using Quiagen Plasmid Kit (Quiagen CA). For RNA synthesis, the plasmid will be linearized and run off transcripts will be prepared by in vitro transcription with the T7 polymerase (Epicentre Technologies, MA). The resulting complementary RNA (internal standard) product will be purified by Chroma Spin Columns (Clontech CA, USA) and the concentrations will be measured at 260nm. Denaturing PAGE will be performed to determine the quality of the cRNA. Competitive RT-PCR will be performed by utilizing four parallel reactions with constant amounts of total RNA isolated from cell lines of interest with increasing amounts of internal standard RNA (0.125pg to 100pg). The two PCR products of differing size will be analyzed using agarose gel electrophoresis. After densitometric evaluation of band intensities of both amplified products the absolute amount of initial template will be calculated. These experiments will be run in triplicate.

Gel Shift Assay (EMSA): Nuclear extracts are prepared by 0.45M KCl extraction. Electrophoretic mobility assays are performed using standard conditions.¹¹ DNA binding reactions will be performed as described.^{12,13} Nuclear extracts are incubated with 10 fmol of the ³²P labeled CBFA1 binding site consensus oligonucleotide (5'-CGAGTATTGTGGTTAATACG-3') as the probe and nonspecific competitor DNA. Protein-DNA complexes are resolved on a 4% non-denaturing polyacrylamide gel. Anti-sera supershift experiments will utilize polyclonal anti serum directed to a CBFA1 peptide containing 17 amino acids¹³ or preimmune serum (control). (Gift of Dr. Scott Hiebert, Vanderbilt Medical Center Nashville TN/ and Oncogene Research Products, Cambridge, MA).

Probe Synthesis for In Situ Hybridization, Northern Analysis, and Ribonuclease Protection Assay (RPA): Primers were designed against the CBFA1 exon 7 sequence obtained from the GenBank. The forward primer is a 20mer (950-969) and the reverse primer is a 20mer (1324-1305). The expected PCR product is 375bp. RNA was extracted from the MG-63 human osteosarcoma cell line, DNAsed, reverse transcribed and amplified using these primers. The amplicon was then gel purified using a Qiaex II kit. The gel-purified product was kinased and inserted blunt ended (using EcoRV) into phosphatased pBSSKII. After transforming bacteria, the colonies were screened using blue/white selection. The white colonies were screened for insert by restriction digest and the insert has been sequenced in both directions, verifying that its sequence is identical to the 375bp amplicon of CBFA1 exon 7. The construct is called pCBFA1, and has an orientation such that the T3 promoter of pBSSK produces the anti-sense strand and the T7 promoter gives the sense strand. Transcription reactions were done to confirm the proper size. This construct is being utilized for in situ hybridization, Northern and RNase protection assays. We have successfully used this strategy to engineer other cDNA constructs.

Osteocalcin Radioimmunoassay (RIA): Human osteocalcin concentrations in conditioned medium samples (1,3,5,7 days) were measured by radioimmunoassay using rabbit anti-monkey osteocalcin (first antibody) and goat anti- rabbit IgG (second antibody).

Western Blot: Cultures were rinsed with PBS, scraped into buffer and collected by centrifugation. Cells were lysed with hypotonic buffer in the presence of protease inhibitors. Nuclei were collected by centrifugation and resuspended in hypertonic buffer and protease inhibitors. Nuclei were extracted on ice, insoluble material was cleared by centrifugation and soluble proteins were stored at -80°C . Nuclear extracts (30 μg protein per lane) were resolved with 8% SDS-PAGE and electroblotted onto nitrocellulose membrane. Membranes were incubated with a 1:100 dilution of antibody in Tris-buffered saline containing 1% BSA. Affinity purified antibodies specific for CBFA1 were used in these studies. Membranes were incubated with secondary antibody, and protein bands were visualized by streptavidin-HRP chemiluminescence (Pierce, IL).

Results: Human breast adenocarcinoma cell lines have been established in the laboratory and examined by RT-PCR, Northern analysis, immunohistochemistry, and radioimmunoassay to establish the level of expression of osteoblast-specific genes. The focus has been maintained on the MDA-MB-231 adenocarcinoma line, based on our success in obtaining osteolytic metastases with this same line in nude mice (see Technical Objective #3 below).

RT-PCR. (Figure 1) Semi-quantitative PCR was performed comparing the expression of CBFA1 and osteocalcin between HMEC, MDA-MB 231 cell line, and M1. These results were normalized to GAPDH for each sample. There was a two fold increase in both CBFA1 (375bp) and osteocalcin (294bp) in the M1 clone (lanes 5 and 2) compared to the original MDA-MB 231 cell line (lanes 4 and 1). There was no evidence of CBFA1 in the HMEC (lanes 3 and 6) cell line. PCR product identity was confirmed by producing products of expected size. The CBFA1 amplicon was excised from the gel, purified and underwent automated sequencing to further confirm its identity.

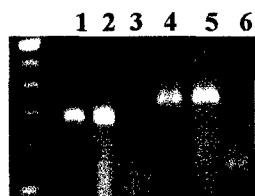


Figure 1

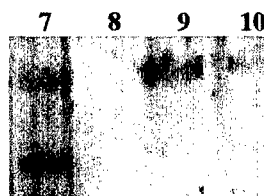


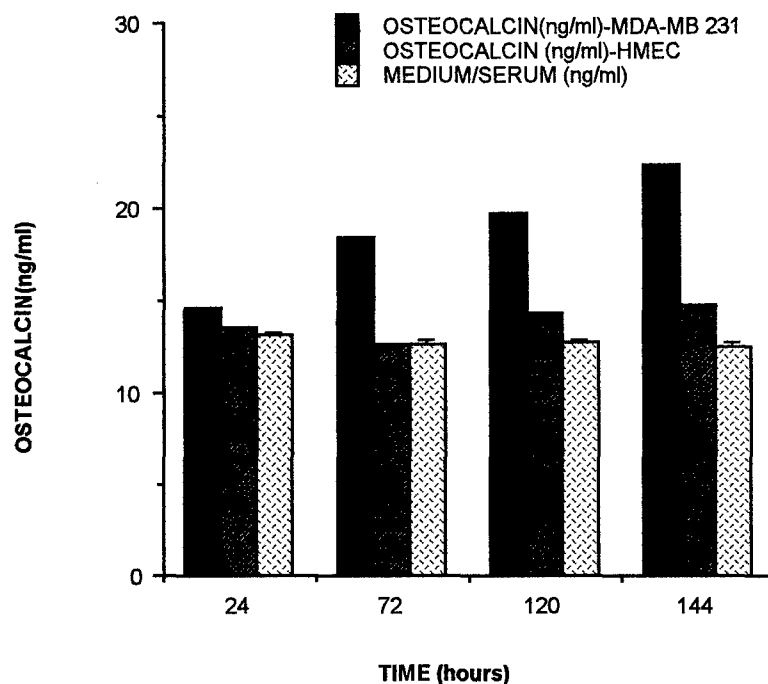
Figure 2

Western Blot: (Figure 2) We examined nuclear extracts from the MDA-MB 231 cell line, HMEC line, and M1 subclone, and MG-63 osteosarcoma cell line (positive control) using antibody to CBFA1. CBFA1 was detected in all of these cell lines except the normal breast line HMEC (Lane 8). A 60-65 kDa species was present in the MDA-MB 231 cells (Lane 10) and M1 subclone (Lane 9). The species was present at a two-fold greater band density in the M1 clone (Lane 9). The MG-63 human osteosarcoma cells (Lane 7) expressed the 60-65 kDa form as well as a prominent 46 kDa form that was not seen in the breast cancer cell lines.

Breast Cancer Cells Express Increased Levels of Osteocalcin Compared to Normal Breast Tissue: Human osteocalcin concentrations in conditioned medium samples of the MDA-MB 231 cells line and HMEC cell lines were measured by radioimmunoassay using a specific osteocalcin antibody. Data are reported as ng osteocalcin/ml of medium. Medium (with 10% FCS) served as

the baseline control, and this accounts for the background which is 13 ng/ml of cross reacting bovine osteocalcin contributed by the 10% serum (FCS). MDA-MB-231 cells expressed increasing amounts

Figure 3



of osteocalcin with increasing confluence. The HMEC cell line did not produce osteocalcin when compared to medium controls. However, other less osteotropic cell lines have not yet been examined to determine if there is an association between osteocalcin production and ability to metastasize to bone. Future experiments will examine less osteotropic breast cancer cell lines, non-osteotropic and non breast malignancies (i.e. colon cancer), as well as the M1 and the LC115 bone metastasis derived cell lines, in order to corroborate the apparent positive correlation between high osteotropism and expression of CBFA1 and osteocalcin.

Electromobility Shift Assays (EMSA): Nuclear extracts prepared from cultured breast adenocarcinoma and normal cell lines have been subjected to EMSA for analysis of CBFA1 transcription factor activity. This analysis utilizes a target oligonucleotide containing the CBFA1 binding site common to a number of osteoblast-specific gene promoters. The labeled oligonucleotide was shifted to slower mobility by putative CBFA1 activity in MDA-MB-231, M1, M2, Blast-4, with MG-63 osteosarcoma serving as a positive control. EMSA showed the expected competition with excess cold target oligonucleotide.

Task 4: Study the action of hOP-1 in regulating adenocarcinoma cells.

Tumor cell culture with hOP-1 showed decreasing osteocalcin after day 7 by preliminary RIA experiments. This important observation needs to be repeated, and we will also examine proliferation

effects, as hOP-1 also appears to slow the tumor cell growth *in vitro*. In designing the future experiments, we have obtained two new sources of human osteoblasts [FOB (Mayo Clinic) and NHOst (Clonetics)], along with the normal human breast epithelial line, HMEC (which is negative for osteocalcin).

Progress on Technical Objective #2

Technical Objective 2: Study the bidirectional interaction between breast adenocarcinoma cells and bone cells (osteoblasts and osteoclasts) *in vitro*.

Methods: Add tumor cells to established cultures of normal osteoblasts for direct contact co-culture, and search for changes in gene expression and proliferation of the tumor cells which require cell contact or labile products of living osteoblasts.

Results: Co-culture experiments with trans-well configurations of MDA-MB-231 cells and MC3T3-E1 cells were fraught with difficulties. The principal problem is the rapid growth of the tumor cells and the resulting nutrient depletion and medium acidification which adversely affects the slower-growing osteoblasts. These experiments were discontinued in favor of direct co-culture. It required much trial and error to determine the appropriate plating densities and culture conditions. The optimal conditions for co-culture are established for 24-well plates: 1) plate 1.2×10^5 MC3T3 cells (day 0); 2) overlay 1.2×10^4 MCF-7 cells when osteoblasts cover 80% of the culture surface (day 2); 3) fix at day 4 or later for immunohistochemistry (Table 1).

Table 1. Immunohistochemistry of Breast Adenocarcinoma (MCF-7) and Osteoblastic (MC3T3) Cells

Antigenic Marker	Cells Cultured Alone		Cells Co-Cultured (2d)	
	MCF-7	MC3T3	MCF-7	MC3T3
human cytokeratin-18	+++	—	++++	—
bone sialoprotein (BSP)	++	—	+++	—
osteopontin (OPN)	++	—	+++	—
osteocalcin (OC)	+	±	++	±
alkaline phosphatase	—	+++	±	+++
non-immune serum control	—	—	—	—

Immunohistochemistry: Table 1 shows the results of antibody staining of breast adenocarcinoma (MCF-7) and osteoblastic (MC3T3) cells alone and in co-culture. The human-specific cytokeratin-18 antibody clearly distinguishes the epithelial adenocarcinoma cells from the murine osteoblasts, as expected. The osteoblasts are selectively stained by alkaline phosphatase (BCIP reaction), and weakly for osteocalcin, as expected for this early time point in the culture (day 4). What is important about the data in Table 1 is the relatively strong expression of the bone cell markers by the MCF-7 cells, and the observation that the staining increases when the MCF-7 tumor cells are co-cultured with the MC3T3 osteoblasts. The growth behavior of the MCF-7 cells in co-culture is in isolated clonal clusters containing 5-50 cells which are surrounded by flattened osteoblasts. The density of the positive immunohistochemical staining for the proteins such as BSP and OPN is greater at the periphery of these clusters, suggesting either a contact-dependent

regulation of gene expression, or a possible uptake of osteoblast-produced proteins by the tumor cells. These possibilities will be discriminated by future experiments employing in situ hybridization and species-specific immunostaining.

Bidirectional Interactions of Tumor Cells and Osteoblasts: It is noteworthy that when larger numbers of MCF-7 cells are plated on the osteoblasts (i.e., when the ratio of tumor cells/osteoblasts is greater than 1/2, then the osteoblasts lift off the culture surface and die within 3 days. Even if the medium is buffered by HEPES to prevent overt acidification by the metabolically active tumor cells, rounding and detachment of the osteoblasts is only delayed by 1-2 days.

Conditioned medium experiments showed that both MCF-7 and MC3T3 cells produce attachment factors which increase each other's attachment to plastic in serum-free conditioned alpha-MEM. However neither of the cell types can survive beyond 48-72 hr in these conditioned media, presumably because of the absence of serum growth factors. At this time, we have no evidence of overt cytotoxic factors being produced by either cell type for action on the other.

Progress on Technical Objective #3

Technical Objective 3: Establish human breast adenocarcinoma tumors at primary and skeletal metastatic sites by inoculation of athymic nude mice. Analyze the gene expression of tumor and bone cells in the recovered tissue. Test the activity of shOP-1 on primary tumor growth and on establishment and progression of skeletal metastases.

Task 9: Establish the skeletal metastasis model, first with MCF-7, and then with the more aggressive MDA-MB-231 line. Perform a cell inoculation dose study and monitor osteolysis in developing skeletal metastases radiographically. Recover metastases for histology. Compare gene expression patterns by cells at the tumor/bone interface with tumor harvested from soft tissue (eg., lung).

Methods: Animals: 7 week old, female Nu/Nu SCID mice (20-25g) were used following institutional guidelines for the use and care of laboratory animals. MDA-MB 231 cells were injected (1×10^5 cells/100 μ l) through the left second intercostal space into the left ventricle to produce bone metastases. Animals were radiographed bimonthly to detect the presence of lytic lesion. Bones with lesions were fixed in 4% paraformaldehyde and decalcified. Several lesions were isolated and subcultured to produce a breast cancer cell lines from a bone metastasis. These subclones were further characterized by immunostaining with cytokeratin 18 (mouse anti-human) to confirm both the human and glandular epithelial origin. Reinjection into mice was performed to confirm malignancy. Immunohistochemistry: Monoclonal or polyclonal antibodies to osteocalcin and CBFA1 and appropriate negative controls were employed. The sections were obtained from the distal femur and proximal tibia of mice. Archival human specimens from 10 patients with metastatic breast cancer to appendicular and axial sites were similarly examined. Fixed, paraffin embedded sections from each tumor were immunostained using an indirect avidin-biotin complex method. The appropriate concentrations of the primary antibodies against CBFA1 and osteocalcin were used (1:10 and 1:100, respectively).

Results:

One year of experience with the mouse model for osteolytic metastasis of breast cancer has shown that approximately 20% of mice develop metastases which are osteolytic by radiographic

criteria. In about 2-5% of mice, multiple osteolytic sites are found. The lead orthopaedic surgeon on this project, Andrew Hecht, M.D., has recently traveled to San Antonio, TX in order to receive further training in this model from the Yoneda/Mundy laboratory.

Recovery of Malignant Human Breast Adenocarcinoma Cells from Bone Metastases: Osteolytic bone metastases (and one osteosclerotic metastasis) were located radiographically in mice which had been injected by the intracardiac method with MDA-MB-231 cells. At sacrifice, 15 sites were dissected and curetted to obtain cells for tissue culture. Of these candidate cultures, 2 osteolytic ones (M1 and M2) grew vigorously and were verified to be sublines of the human MDA-MB-231 adenocarcinoma by human-specific cytokeratin-18 immunostaining which showed human, glandular epithelial origin. The osteosclerotic metastasis yielded a subline (Blast-4) which is also derived from the parental MDA-MB-231 line.

Characterization of Osteoblast Gene Expression Patterns in Human Breast Adenocarcinoma Cells from Osteolytic and Osteosclerotic Metastases:

Immunohistochemical Investigation of Metastases Recovered from Mice: looked for CBFA1 in osteolytic tumor metastases and found positive evidence of the expression of this transcription factor in tumor foci, with little or no expression in mature bone itself. The CBFA1 expression by osteoblasts is primarily confined to the rapidly growing regions of the skeleton (growth plates, etc.). Osteocalcin expression was observed in the tumor foci more strongly than in the surrounding bone. We have started in situ hybridization experiments to look for osteocalcin and CBFA1 mRNAs, but no conclusive results have yet been obtained due to problems with significant background autofluorescence in the bone matrix. An ³⁵S-riboprobe approach may be necessary.

Gene Expression: The small size of the human breast adenocarcinoma metastases in mice makes it impossible to perform certain types of molecular analysis, so we have resorted to analyzing the adenocarcinoma cell lines (M1, M2, and Blast-4) recovered from individual metastases. M1 and M2 are positive for CBFA1 expression RT-PCR (Fig.1), EMSA and Westerns (Fig.2). Northern analysis for CBFA1 has been problematic due to comigration of CBFA1 mRNA with the abundant 18s RNA. Blast-4 cells are positive for CBFA1 by EMSA and Western blotting. In summary, we have the parental line (MDA-MB-231), 3 recovered sublines (M1, M2, and Blast-4), and another MDA-MB-231 subline recovered by another laboratory (C5; M. Tondravi). All 5 of these cell lines express CBFA1, while normal breast cells (HMEC) do not.

Analysis of Archival Human Specimens: Archival human specimens have been obtained from 20 patients with metastatic breast cancer. The primary breast lesions and bone metastases have been obtained from the records of the Orthopaedic Oncology Service at the Massachusetts General Hospital. Paraffin block specimens from metastases in the femur have been obtained. Both osteocalcin and CBFA1 were noted to be positive for immunostaining in both archival human specimens and in experimentally induced animal models of bone metastasis. CBFA1 was seen primarily in the breast cancer cells within each metastasis, with minimal staining of the surrounding bone. Osteocalcin was seen in both the breast cancer metastasis and surrounding bone in both the archival human and animal specimens.

Discussion:

Preliminary data to date suggest that the breast adenocarcinoma cell expresses both osteocalcin and CBFA1. There appears to be increased expression of osteocalcin and CBFA1 in the bone metastasis-derived cell line (M1) when compared to the original MDA-MB-231 tumor line, whereas the normal human breast HMEC cells are negative for both markers. These observations suggest a strong correlation between increasing metastatic potential and the expression of the osteoblast phenotype by the adenocarcinoma cell.

Microcalcification of Breast Lesions: A Possible Consequence of Osteoblastic Mimicry by Adenocarcinoma Cells? An important clinical observation suggesting the existence of a privileged relationship between breast and bone tissue is that deposits of calcium compounds are often seen radiographically and histopathologically among breast carcinoma cells. Ectopic calcifications associated with malignant lesions are formed by hydroxyapatite, $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$, the basic mineral found in the skeleton. Microcalcifications are an important indication of early breast carcinoma.¹⁴ In many instances, the detection of microcalcifications on mammography is the unique sign indicative of the presence of a breast lesion. However, microcalcification deposits in the breast are not restricted to malignant lesions, but can also be associated with benign conditions such as fibroadenoma, secretory diseases and fat necrosis.¹⁵ Ultrastructural analysis of breast cancer associated microcalcifications has revealed crystalline deposits in the cytoplasm of malignant cells calcification of cytoplasmic organelles, and membrane bound vesicles with hydroxyapatite crystals.¹⁶ These observations have suggested that hydroxyapatite deposition within breast cancer results from an active mechanism rather than the mineralization of cellular debris and necrotic material. Although mammographically detected microcalcifications are frequently the only sign of malignant breast tissue, the mechanism of their deposition has not yet been elucidated. Apparently, breast cancer cells are able to generate a microenvironment that promotes crystallization of calcium and phosphate into bone-like mineral. This observation led investigators to examine breast cancer cells for the expression of bone matrix proteins. Osteonectin, osteopontin and bone sialoprotein have been studied in a series of human breast cancers.^{3,15-30} Immunohistochemical evaluation of these three proteins in benign and malignant breast lesions revealed markedly increased expression of BSP/OPN/OSN when compared to benign lesions or normal breast tissue. The detection of OSN and OPN in breast tissues was not surprising due to the ubiquitous presence of these two proteins in many normal and cancerous tissues of the body,³¹ however, the expression of BSP was thought to be strictly limited to mineralized connective tissues. BSP was found to be present in numerous breast adenocarcinoma cell lines, both estrogen dependent and independent. In addition, BSP expression was found to be a poor prognostic sign when present in the primary lesion. There was a significantly increased incidence of subsequent bone metastases in patients who expressed higher levels of BSP.¹⁸

Other matrix proteins may also be important in the metastatic process. Osteocalcin has been implicated, along with BSP, in osteoclast recruitment, activation and attachment. However, there are virtually no reports of non-osteoblastic osteocalcin expression. Our preliminary findings of osteocalcin expression by MDA-MB-231 cells identifies osteocalcin as a potential regulator of osteolytic metastasis.

CBFA1, the Transcriptional Regulator of the Osteoblast Phenotype: The molecular mechanisms involved in the selective interaction of breast cancer cells with the bone matrix are not yet identified. The finding that the minor bone proteins; BSP, OPN and OSN, may play a role in this metastatic process may be further illuminated by the discovery of a novel transcription factor that

controls osteoblast differentiation. A transcription factor, CBFA1, was recently shown to control the expression of many osteoblast-specific genes. CBFA1 (core-binding factor), also referred to as PEB2A α (polyoma enhancer-binding protein) and AML-3 (acute myelogenous leukemia) is one of three mammalian genes (i.e. CBFA1, CBFA2, CBFA3) which encode transcription factors whose DNA-binding domains share homology with the *Drosophila* segmentation gene product runt.³² The alpha subunit of these heterodimeric protein transcription factors binds to DNA via the runt domain when paired with the β -subunit which does not directly interact with the DNA. Recent studies have shown that CBFA1 controls the pathway of differentiation into the osteoblast lineage. The promoter regions of the osteoblast phenotype related genes including OPN, osteocalcin, BSP, and type I collagen contain the core binding sequence.³³⁻³⁷ Deletion of the CBFA1 gene in mice leads to a total absence of osteoblasts owing to an arrest in their differentiation.^{38,39} Komori et al. and Otto et al. independently performed a similar study involving the creation of a CBFA1 knock-out mouse.³⁷⁻³⁹ The homozygous CBFA1 $-/-$ mice died soon after delivery, cyanotic, due to inability to breathe. The homozygotes were smaller and had shorter limbs, but all remaining organs were proportionate. The most striking finding was the total lack of bone and retention of the partially calcified cartilaginous skeleton. Membranous bones of the skull and endochondral bone in the skeleton were absent. The tibia contained only calcified cartilage where bone is usually formed at this age. Histology revealed an absence of osteoblasts and smaller sized osteoclasts. Investigators performed northern blot analysis on a number of genes associated with the osteoblast phenotype: osteocalcin, BSP, OPN and alkaline phosphatase. Expression of these genes was greatly diminished. Normal control animals revealed that CBFA1 gene expression was localized during development (via in situ hybridization) to regions destined for bone development. Osteoblast gene expression was examined after BMP-2 stimulation. Both osteocalcin and alkaline phosphatase gene expression was markedly reduced in the mutant calvaria-derived cells. Similarly the genes normally expressed in bone (osteocalcin and osteopontin) were suppressed in CBFA1 $-/-$ mice.

CBFA1 maps to mouse chromosome 17 in mouse and to and to 6p21 in humans^{40,41} at the same location as cleidocranial dysplasia (CCD). Otto³⁹ also noticed that there were abnormalities in heterozygous mice, most prominently: hypoplasia of the clavicle, delayed development of membranous bones, and delayed ossification of cranial bones, causing open anterior and posterior fontanelles, smaller parietal and interparietal cranial bones, and multiple Wormian bones. These features suggested a possible similarity with the human clinical correlate of cleidocranial dysplasia syndrome (CCD). The human CCD syndrome, an autosomal dominant disorder exhibits all the features described above in the CBFA1 $+/-$ mice, plus supernumerary teeth.⁴²

CBFA1 was capable of inducing osteoblast related genes in non-osteoblastic tissue. In C3H10t1/2 cells and in skin fibroblasts, transient transfection with a CMV-promoter driven CBFA1 construct up-regulated expression of OPN, osteocalcin, and type I collagen. This essential transcription factor thus controls the lineage specific differentiation of osteoblasts, and may control the breast adenocarcinoma mimicry of the osteoblast phenotype.

This study is examining the role of CBFA1 in controlling the osteoblast phenotype in the metastatic breast adenocarcinoma cell. It is becoming clear that non-osteoblastic cells may be capable of expressing this protein, and it may have different functions in different cell types.^{37,43} The unique bone protein osteocalcin is also being examined, as CBFA1 directly controls osteocalcin expression. The presence of osteocalcin is indicative of the osteoblast phenotype and this may signify that other key members of the osteoblast program (i.e. PTH/PTHrP receptor, BSP, OPN) are also being expressed by the tumor cells, and that these may also be under the control of CBFA1. The

expression of the osteoblast program by the adenocarcinoma cell may allow tumor cells to override the normal osteoblast-osteoclast relationship and to pathologically induce the hormone dependent and cytokine dependent driving of the osteoclast that leads to osteolysis.

Conclusions (Importance and Implications)

Bone is the most common site of invasive breast cancer metastasis (over 90% of women dying of breast cancer have bone metastases). The extreme morbidity caused by skeletal metastases (vertebral compression fracture, paraplegia, long bone fracture, severe bone pain) is a major consequence of this disease, causing great personal suffering while consuming an estimated 63% of the total costs of caring for patients with recurrent breast cancer. About 44,300 women died from breast cancer in the U.S. in 1996, and some 184,300 new cases of the disease were diagnosed.^{44,45}

The innovative approach of this project is that it focuses on defining the limited number mechanisms by which breast adenocarcinoma cells interfere with normal cellular communication in the host skeleton. These mechanisms involve inappropriate ectopic expression of bioactive bone proteins which activate osteoclasts (PTHrp, bone sialoprotein, osteopontin), expression of integrins allowing attachment to the bone ECM, and unregulated growth factor expression. All clinical protocols which target breast cancer with hormone therapy and cytotoxic chemotherapy must contend with a frustrating biological fact: heterogeneity of the malignant adenocarcinoma phenotype. Diverse mutations and clonal selection of variants evolving in independent sites over the progression of the disease in each patient create an evasive target for chemotherapy. We anticipate that regardless of their detailed phenotypic profile, these malignant cells colonize bone by essentially the same process, and our findings will thus provide a rationale for blocking bone metastasis of breast cancer.

Predictors of future metastasis are of great importance in selecting the high-risk group of *node-negative* breast cancer patients needing careful observation and potentially benefitting from adjuvant therapies. Just as emerging data for microvessel density (angiogenesis) in primary sites correlate well with future recurrence, so may bone protein marker expression eventually predict skeletal metastasis and allow early intervention.

The expected outcome of this Project is the development of molecular approaches to dismantle the pathways by which normal bone cells are coerced into nurturing metastatic cells in the skeletal microenvironment. Importantly, these pathways should be relatively stable targets for therapeutic intervention, in contrast to the highly mutable phenotypes of breast adenocarcinoma cells.

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Appendix**Abstract to 45th Orthopedic Research Society, February 1-4, 1999, Anaheim, CA
Accepted for oral presentation.****Breast Cancer Metastases to Bone Express Increased CBFA1 and Osteocalcin.**

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Danciu T, Keel S, Skazkina K, Gebhardt MC, and Hauschka PV.

Introduction: Bone Metastases are common in breast cancer. Large autopsy studies estimate their frequency at 85%. Complications of bone metastases include pain, pathologic fracture, loss of mobility and vertebral compression syndromes. The osteotropism of breast cancer metastases remains poorly understood. Several investigators have demonstrated that breast cancer cell lines as well as primary and metastatic tumors synthesize bone proteins such as osteopontin (OPN) and bone sialoprotein (BSP). The transcription factor, CBFA1, regulates the expression of the osteoblast related genes (OC, OPN, BSP). We examined the expression of the osteoblast transcription factor CBFA1 and its target gene osteocalcin (OC) in the MDA-MB 231 cell line, a subclone of this line isolated from a bone metastasis (M1), and archival human specimens of patients with bone metastases from breast cancer primaries. Our central hypothesis was to determine if the expression of the osteoblast phenotype correlated with increased metastatic potential.

METHODS: Cell Lines: MDA-MB 231 (ATCC, MD) and Human Mammary Epithelial Cell (HMEC) (Clonetics, CA) were cultured according to recommended guidelines. The M1 subclone was cultured under conditions identical to the MDA-MB 231 cell line. Animals: 7 week old, female Nu/Nu SCID mice (20-25g) were used following institutional guidelines for the use and care of laboratory animals. MDA-MB 231 cells were injected (1×10^5 cells/100ul) through the left second intercostal space into the left ventricle to produce bone metastases. Animals were radiographed bimonthly to detect the presence of lytic lesion. Bones with lesions were fixed in 4% paraformaldehyde and decalcified. Several lesions were isolated and subcultured to produce a breast cancer cell line from a bone metastasis (M1). This subclone was further characterized by immunostaining with cytokeratin 18 (mouse anti-human) to confirm both the human and glandular epithelial origin. Reinjection into mice was performed to confirm its malignancy.

RNA/RT PCR RNA extraction was performed using RNeasy Mini Kit (Quiagen, CA) Primers were synthesized to unique regions in the osteocalcin, CBFA1, and GAPDH human cDNA sequences reported in the Genbank. PCR reaction cycle conditions consisted of denaturation at 93°C for 60s, primer annealing at 61°C for 60s, extension at 72°C for 60s, and a final extension at 72°C for 7 min in a Perkin-Elmer thermocycler (Model 9600). Samples were amplified for 25 cycles. UV photography and densitometry of ethidium bromide stained gels was performed. Semi-quantification was achieved by comparing the intensities of both CBFA1 and osteocalcin to GAPDH.

OC radioimmunoassay. Human OC concentrations in conditioned medium samples (1,3,5,7 days) were measured by radioimmunoassay using monkey anti-human osteocalcin (first antibody) and goat anti-monkey osteocalcin (second antibody). Data are reported as nanograms of OC/ml of medium. Alpha MEM with 10% FCS was used as a control.

Western Blot: Cultures were rinsed with PBS, scraped into buffer and collected by centrifugation. Cells were lysed with hypotonic buffer in the presence of protease inhibitors. Nuclei were collected by centrifugation and resuspended in hypertonic buffer and protease inhibitors. Nuclei were

extracted on ice, insoluble material was cleared by centrifugation and soluble proteins were stored at -80°C . Nuclear extracts (30ug per lane) were resolved with 8% SDS-PAGE and electroblotted onto nitrocellulose membrane. Membranes were incubated with a 1:100 dilution of antibody in Tris-buffered saline containing 1% BSA. Affinity purified antibodies specific for CBFA1 (gift from Dr. Scott Hiebert, Vanderbilt Cancer Center, Nashville, TN) were used in these studies. Membranes were incubated with secondary antibody followed by streptavidin-HRP chemiluminescence (Pierce, IL).

Immunohistochemistry: Monoclonal or polyclonal antibodies to OCN and CBFA1 and appropriate negative controls were employed. The sections were obtained from the distal femur and proximal tibia of mice. Archival human specimens from 10 patients with metastatic breast cancer to appendicular and axial sites were similarly examined. Fixed, paraffin embedded sections from each tumor were immunostained using an indirect avidin-biotin complex method. The appropriate concentration of the primary antibody against CBFA1 and OC were used (1:10 and 1:100).

Results: RT-PCR. (Figure 1) Semi-quantitative PCR was performed comparing the expression of CBFA1 and OC between HMEC, MDA-MB 231 cell line, and M1. These results were normalized to GAPDH for each sample. There was a two fold increase in both CBFA1 (375bp) and OCN (294bp) in the M1 clone (lanes 5 and 2) compared to the original MDA-MB 231 cell line (lanes 4 and 1). There was no evidence of CBFA1 in the HMEC (lanes 3 and 6) cell line. PCR product identity was confirmed by producing products of expected size. The CBFA1 amplicon was excised from the gel, purified and underwent automated sequencing to further confirm its identity.

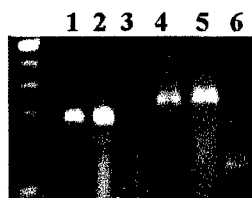


Figure 1

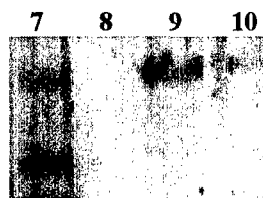


Figure 2

Western Blot: (Figure 2) We examined nuclear extracts from the MDA-MB 231 cell line, HMEC line, and M1 subclone, and MG-63 osteosarcoma cell line (positive control) using antibody to CBFA1. CBFA1 was detected in all of these cell lines except the normal breast line HMEC (Lane 8). A 60-65kDa species was present in the MDA-MB 231 line (Lane 10) and M1 subclone (Lane 9). The species was present at a two-fold greater band density in the M1 clone (Lane 9). The MG-63 line (Lane 7) expressed the 60-65 kDa form as well as a 46 kDa form that was not seen in the breast cancer cell lines.

Radioimmunoassay: MDA-MB 231 cells produced increasing amounts of osteocalcin. OC concentrations from medium + serum control was measured at 12.8 ng/ml. OC from tumor conditioned medium revealed increasing osteocalcin concentrations. Day 1 produced 14.6 ng/ml; Day 3, 18.4ng/ml, Day 5, 19.7 ng/ml; Day 7, 22.4 ng/ml.

Immunohistochemistry. Both osteocalcin and CBFA1 were noted to be positive for immunostaining in both archival human specimens and in experimentally induced animal models of bone metastasis. CBFA1 was seen primarily in the breast cancer metastasis, with minimal staining of the surrounding bone. Both intra and extracellular staining was noted. CBFA1 was noted to be only in the breast cancer metastasis in the archival human specimens with no staining in the surrounding bone. OC was seen in both the breast cancer metastasis and surrounding bone in both the archival human and animal specimens.

DISCUSSION: Several studies have found correlations between an increase in osteonectin, OPN and BSP expression with the degree of breast cancer invasiveness. In previous studies, the osteoblast transcription factor CBFA1 has not been found in non-skeletal tissues. CBFA1 plays a pivotal role in osteoblast differentiation and bone formation. This study reveals for the first time that invasive breast cancer is able to synthesize CBFA1 and OC. In addition adenocarcinoma cells isolated from bone expressed a two fold increase in both CBFA1 and OC. The expression of bone related genes may have implications in the pathologic destruction of bone as well as in its osteotropism. OSTEOCALCIN has been shown to increase the recruitment of osteoclasts and promote bone resorption. Paget in 1889 proposed that implantation of a given population of circulating cancer cells at a selected site of the organism is dependent on a suitable environment ("the soil") in which compatible tumor cells ("the seed") could proliferate. The molecular mechanisms underlying this well accepted theory remain poorly understood. The expression of the osteoblast phenotype by the breast cancer cell may give it a survival advantage in the bone microenvironment. In addition, CBFA1 mediates both BSP and OPN expression. These proteins may facilitate the targeting and attachment of circulating tumor cells to areas of ossification. It is tempting to speculate that the expression of CBFA1 may play a key role in explaining the increased osteotropic phenomenon of breast cancer, as well as breast cancer microcalcification,



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